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<p>(54) Title: IMPROVED AGROBACTERIUM-MEDIATED TRANSFORMATION OF PLANTS</p>		
<p>(57) Abstract <i>Agrobacterium</i> strains which have lost the capacity to proliferate vigorously <i>in vitro</i> or <i>in planta</i>, are provided, as well as transformation methods using these <i>Agrobacterium</i> strains.</p>		

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IMPROVED *AGROBACTERIUM*-MEDIATED TRANSFORMATION OF PLANTS

5 This invention relates to the use of *Agrobacterium* strains which have lost the capacity to proliferate vigourously *in vitro* or *in planta*, particularly to the use of auxotrophic *Agrobacterium* strains, to generate transformed plants.

Background of the invention

10 Over the years many techniques for the genetic transformation of plants have been developed. These methods have as ultimate goal to obtain a transgenic plant, in which all cells contain a foreign DNA comprising a gene of interest (the so-called transgene) stably integrated in their genome, particularly their nuclear genome.

15 Different plant transformation methods have been described and can be classified into physical DNA delivery methods (e.g. electroporation, PEG-mediated DNA uptake, biolistics) or *Agrobacterium*-mediated DNA transfer. The latter one frequently is superior in efficiency, simplicity and quality of the transgenic plants (which generally comprise a smaller number of transgenes and have a lower occurrence of aberrant transgenes).

20 *Agrobacterium*-mediated DNA transformation of plants is based on the capacity of certain *Agrobacterium* strains to introduce a part of their Ti-plasmid, i.e. the T-DNA, into plant cells and to integrate this T-DNA into the nuclear genome of the cells. It was found that the part of the Ti-plasmid that is transferred and integrated is delineated by specific DNA sequences, the so-called left and right T-DNA border sequences and that the natural T-DNA sequences between these border sequences can be replaced by

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foreign DNA (European Patent Publication "EP" 116718; 1987 Deblaere *et al.* (1987) *Meth. Enzymol.* 153: 277-293).

5 Frequently, *Agrobacterium*-mediated transformation protocols call for the use of readily regenerable excised plant tissues, organs or parts of organs, such as leaf discs, internodia, stem segments and the like. Alternatively, *in vitro* cultured tissues (e.g. compact embryogenic callus), suspension cultures or single cells (protoplasts) are employed as starting material to be transformed. For *Arabidopsis*, protocols have been
10 described wherein seeds or total plants are inoculated (so called *in planta* transformation protocols).

A common feature of all protocols is that the cells, tissues or plants to be transformed are co-cultivated for a certain time with the *Agrobacterium*
15 strains and subsequently, the proliferation of the *Agrobacterium* strains has to be restricted or eliminated by the use of bacteriocides or bacteriostatics such as antibiotics, particularly if further *in vitro* culturing is required. Frequently, the utilized concentrations of antibiotics interfere with or even inhibit the efficient regeneration of the transformed plant cells. Indeed it
20 has been demonstrated that addition of certain antibiotics with a beta-lactam core structure, such as carbenicillin, to the plant medium may have cytokinin-like effects (WO 97/12512). Furthermore, the *Agrobacterium* strain of interest frequently comprises a bacterially expressed antibiotic resistance gene, such as but not limited to beta-lactamases, further
25 restricting the spectrum of suitable antibiotics.

A problem frequently observed with the transformation of certain recalcitrant plant species, is that the regenerable cell layer may not be readily accessible to *Agrobacterium*-mediated transformation. Indeed,
30 histogenetic analysis has demonstrated that e.g., in tomato, shoots

induced by cytokinin treatment are derived from a few neocambial cells (Monacelli *et al.* 1988, *Protoplasma* **142**, 156-163).

Another regularly encountered problem in *Agrobacterium* mediated transformation is the stress that vigorously proliferating *Agrobacterium* strains exercise on plants or plant parts, particularly when culturing the transformed plants or tissues *in vitro*, which may lead to inhibited regeneration or even death of the cells or explants.

Lippincott *et al.*, 1965 (*Journal of Bacteriology* **90**, 1155-1156) describe that auxotrophic mutants of *Agrobacterium* strain B6 are greatly reduced in infectivity (<1 to 30% of the parent specific infectivity).

Lippincott and Lippincott (1966, *Journal of Bacteriology* **92**, 937-945) describe the further characterization of *Agrobacterium* mutant strains auxotrophic for adenine, methionine or asparagine and established that infectivity could be increased by simultaneously applying the required nutrient to the infected leaves at the time of infection with the auxotrophic *Agrobacteria*.

Christen *et al.* (1984, *Z. Pflanzenphysiol. Bd.*, **113** S. 213-221) demonstrated that leucine- and histidine requiring *Agrobacterium tumefaciens* mutants grow extremely poorly in the presence of dividing *Nicotiana tabacum cv. xanthi* protoplasts and suggested the use of such auxotrophic strains for cocultivation with plant protoplasts in the presence of a limiting amount of the required nutrient to allow temporary *Agrobacterium* division, as an alternative to the use of antibiotics.

Chang *et al.* (1994, *The Plant Journal* **5**, 551-558) describe a transformation protocol for *Arabidopsis* involving severing of apical shoots at their bases, inoculation with *Agrobacterium* at the severed sites, and *in*

planta generation of shoots from the severed sites to produce stably transformed progeny.

5 An improved *in planta* transformation method for *Arabidopsis* was described by Bechtold *et al.* (1993, *C.R. Acad. Sci. Paris, Sciences de la vie* 316: 1194-9) based on vacuum infiltration of a suspension of *Agrobacterium* cells into *Arabidopsis* plants, followed by selection of transformed progeny in the T1 seed. The authors assumed that a main limiting factor for the transformation frequency would be the restricted
10 persistence of bacteria in the plant from the germination stage until the seed formation.

Summary of the invention

15 In accordance with the invention, provided is a process to produce a transgenic plant comprising a foreign DNA fragment integrated into the genome of at least some of its cells, comprising the following steps:

- 20 1) providing a plant or part of a plant, which is systemically infected with an auxotrophic *Agrobacterium* strain, preferably a methionine or cysteine requiring or a histidine and adenine requiring auxotrophic *Agrobacterium* strain, particularly LBA4404*met* or ATHV*ade,his*, harbouring a DNA of interest which is linked to at least one T-DNA border sequence so as to be capable of being transferred to a plant cell, wherein the
25 infected plant is preferably regenerated from explant tissue inoculated with said auxotrophic *Agrobacterium* strain ; and
- 2) generating a transgenic plant from a single cell or a group of cells isolated from the systemically infected plant, preferably by *in vitro* regeneration of an explant tissue, a
30 protoplast or a microspore isolated from the systemically

infected plant or alternatively by germination of transformed progeny seed of the systemically infected plant.

5 The process may further comprise the step of applying the required nutrient to at least part of the systemically infected plant, preferably to the inflorescence meristem or immature inflorescence of the systemically infected plant, prior to the step of generating a transformed plant from the systemically infected plant.

10 Also provided by the invention are *Agrobacterium* strains auxotrophic for methionine or cysteine or auxotrophic for adenine and histidine, particularly LBA4404*met* or ATHV*ade,his*, and their use in *Agrobacterium*-mediated transformation of plants, particularly in *Agrobacterium* mediated transformation of corn protoplasts, or alternatively in the *Agrobacterium*-
15 mediated transformation of embryogenic callus, particularly embryogenic callus from cucumber or sugar beet.

Detailed description of the invention.

20 The present invention is based on the finding that auxotrophic *Agrobacterium* strains as hereinafter defined can survive when inoculated on an explant and successfully establish a systemic infection of the regenerated plant. The presence of the endemic auxotrophic *Agrobacteria* does not interfere with the plant regeneration, even in the
25 absence of bacteriocidal or bacteriostatic compounds. Moreover, such bacteria are still able to transform cells, as can be evidenced by the detection of transformed patches of marker gene expression in the regenerated plants. Finally, tissue explants from the systemically infected plants can be further cultivated *in vitro*, without bacteria overgrowing the
30 explants.

The auxotrophic *Agrobacterium* strains of the invention allow controlled proliferation of the bacterial cells upon *in planta* and *in vitro* co-cultivation, and inoculated plants, tissues or cells are no longer overgrown by the proliferating bacteria. The *Agrobacterium* strains of the invention can thus be used to establish a plant systemically infected with such strains. The contacting time with the inoculated *Agrobacterium* strain is increased, allowing the bacteria to penetrate the inner plant tissues and reach and transform the appropriate target tissues such as the underlying regenerable cells. Such a prolonged incubation, however, can only be achieved if the vigorous proliferation of the bacteria can be controlled without killing them, as is achieved by the auxotrophic *Agrobacterium* strains of the invention. The method is particularly useful whenever an *in vitro* cultivation step is required in the generation of a systemically infected plant (e.g. regeneration of a systemically infected plant from a tissue explant, e.g. a stem segment).

Furthermore, the use of auxotrophic *Agrobacterium* strains to generate a plant systemically infected with such a strain and combination with the locally supplementing the required nutrient allows the local and controlled growth of *Agrobacteria* inside specifically targetted tissues, such as, but not limited to, the inflorescence yielding meristem.

Auxotrophic *Agrobacterium* strains suited for the methods of the invention, are those *Agrobacterium* strains which are unable to proliferate on a defined medium unless a particularly required nutrient (or nutrients) is (are) supplied to that medium. Preferably, the *Agrobacterium* strain is unable to proliferate vigorously in or on plant tissues.

Particularly preferred auxotrophic *Agrobacterium* strains are auxotrophic for molecules that are present only in limited amounts in plant tissues or intercellular fluids, more particularly for nucleosides or nucleotides (purine or pyrimidine) or amino-acids. Especially preferred is an *Agrobacterium*

strain auxotrophic for an amino-acid, particularly auxotrophic for a sulfur-containing amino-acid, especially auxotrophic for cysteine or methionine. Especially preferred is an *Agrobacterium* strain, that produces a defective homocysteine methyltransferase (EC. 2.1.1.14) or no homocysteine methyltransferase at all. It is however known that mutations in other genes, such as the genes coding for homoserine succinyltransferase (EC 2.3.1.46), cystathionine (gamma)-synthase (EC 4.2.99.9) or cystathionine (beta)-lyase (EC 4.4.1.8), can also lead to an *Agrobacterium* strain auxotrophic for methionine and such strains can be used to similar effect.

Yet another especially preferred *Agrobacterium* strain is an auxotrophic *Agrobacterium* strain requiring two different nutrients, particularly requiring adenine and histidine, such as ATHVade, his.

It is preferred that the auxotrophic *Agrobacterium* mutant strain should have a low frequency of reversion. It is known in the art that deletion mutations result in a low frequency of reversion, and methods to generate deletions in bacteria are available to skilled artisan (e.g. Van Haute *et al.*, 1983, *EMBO J.* 2: 411-417).

It goes without saying that all *Agrobacterium* strains, whether they belong to the species *rhizogenes*, *tumefaciens* or *radiobacter*, can be used according to the invention as host to harbour the T-DNA vectors and helper plasmids necessary to transfer the genes of interest to plant cells, provided they have a mutation rendering them auxotrophic as defined above. A preferred strain is an auxotrophic LBA4404 strain, particularly LBA4404met.

It should also be clear that the type of T-DNA vector or helper Ti-plasmid harboured by the auxotrophic host is not important for working the invention. It has been shown that certain Ti-plasmids such as the

pTiBo542 derived pEHA101 (Hood *et al.* 1986 *J. Bacteriology* **168**: 1291-1301) can improve the frequency of T-DNA transfer to plants. It has also been shown that introduction of the *vir* genes, particularly *virG* or *virG* together with *virB*, of a hypervirulent Ti-plasmid such as pTiBo542, on a replicon which is compatible with the resident Ti-helper plasmid, such as exemplified by pTOK47 (Jin *et al.*, 1987 *J. Bacteriol.* **169**, 4417-4425) can improve the frequency of T-DNA transfer to plants. It is clear that auxotrophic strains harbouring such plasmids can be used for the plant transformation methods of the invention. It has further been demonstrated that so-called superbinary vectors, comprising an extra *virG* gene of a supervirulent Ti-plasmid, particularly a *virG* gene from pTiBo542 can improve the frequency of transfer to certain plants (e.g. EP 0604662) and again auxotrophic strains harbouring such T-DNA vectors can be used for the plant transformation methods of the invention.

In accordance with this invention, a method is provided for producing a transgenic plant comprising a foreign DNA fragment integrated into the genome of at least some of its cells, comprising the following steps .

- 1) providing a plant or part of a plant, systemically infected with an auxotrophic *Agrobacterium* strain harbouring a DNA of interest which is operably linked to at least one T-DNA border sequence, preferably operably linked to two T-DNA border sequences; and
- 2) regenerating a transformed plant from transformed single cells or a group of transformed cells derived from the systemically infected plant.

In a preferred embodiment a systemically infected plant is generated by inoculation of an explant tissue with an auxotrophic *Agrobacterium* strain, as herein described, harbouring a DNA of interest, followed by co-cultivation of the explant and the auxotrophic *Agrobacterium* strain *in vitro* for a period of time sufficient to establish a successful infection. Next, the

excess of bacteria is removed and a systemically infected, intact plant is regenerated.

Methods to cultivate auxotrophic *Agrobacteria* are generally known in the art. It is thought to be important, in order to establish a successful systemic infection, that the required nutrients carried over from the cultivation medium are not completely removed prior to the inoculation step. Furthermore, an incubation of the auxotrophic *Agrobacterium* strain in the presence of plant phenolic compounds inducing *vir*-gene expression, such as e.g. acetosyringone, prior to inoculation, may be included in the methods of the invention. Such pre-induction with plant phenolic compounds has been amply described in the available literature (e.g. Vernade *et al.*, 1988 *J. Bacteriol.* 170: 5822-5829). Alternatively, the *vir*-inducing compound may be supplied locally to the systemically infected plant.

The inoculation method used is expected not to be important. Preferably, the explant is submersed in a suspension of *Agrobacterium* cells with density or OD₆₀₀ ranging from 0.01 to 2, preferably from 0.1 to 1.5, particularly about 0.1. However, other methods such as vacuum infiltration, injection of *Agrobacterium* cells, and the like in plants or plant parts, can be used to the same effect.

It should be clear that it is not necessary that cells of the explant tissue are transformed, just that the explant is infected by the *Agrobacterium* strain, so as to be capable of regeneration into a plant which is systemically infected by the *Agrobacterium* strain.

It is thought that the main limitation for the explant tissue consists in the ability to regenerate into a whole plant, preferably into a fertile plant. Therefore, a wide range of explants, depending on the plant species to be transformed, can be used as starting material for the transformation

methods of the invention, such as stem segments or internodia, callus tissues, preferably embryogenic callus, particularly compact embryogenic callus tissue of cereal plants.

5 In a preferred embodiment the co-cultivation of the explant and the *Agrobacterium* strain *in vitro*, extends for about two to five days, but is it thought that this time period can be shortened to as less as 1-day or extended to as much as 10 days. However, particularly with extended incubation periods, care has to be taken that the *Agrobacteria*, which
10 continue dividing because of the residual required nutrients, carried over from the bacterial cultivation medium upon inoculation, do not completely overgrow the explant.

Excess of bacteria sticking to the surface of the explant, can optionally be removed e.g. simply by washing or dipping on a sterile paper tissue.

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Finally, a plant which is systemically infected with the *Agrobacteria* of interest, is regenerated, either *in vitro*, in soil or both. The method is particularly interesting where a regeneration *in vitro* is required, since the auxotrophic *Agrobacteria* cannot grow on unsupplemented, commonly
20 used plant media (such as e.g. Murashige and Skoog medium) even in the absence of antibiotics.

Transformed plant cells may be enriched during the regeneration of the plant from the explant tissue, by cultivation on media containing a selective
25 agent for the transformed plant cells as generally known in the art. For the method of the invention however it is important that the *Agrobacteria* can tolerate the used selective agent.

It is known that the auxotrophic *Agrobacteria*, which are present in the
30 systemically infected regenerated plants, retain the capacity to transfer the DNA of interest to several tissues, and expression of a transgene has been

demonstrated at least in anthers, leafs, flower primorida and the like. In view of the observed diversity of stably transformed tissue, it is thought that the *Agrobacteria* can gain acces to all cells in all tissues, particularly to the regenerable cells and tissues.

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The regenerated plants will normally consist of a mosaic of transformed and untransformed cells, and it is thought that different patches of transgenic cells may result from independent transformation events. In order to single out the different transformation events, a "clonal" step is preferably included in the process. By "cloning" is meant the process of regenerating a plant starting from single cells or a group of cells. A naturally occuring cloning process is provided by the gametogenesis. Haploid cloned plants can thus be generated e.g. by microspore culture. Homozygous plants with a limited number of transgene insertions can then be readily obtained as "doubled haploids". It is clear however that the gametes can be used to fertilize or be fertilized by a corresponding gamete to yield zygotic embryos.

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Seeds, preferably seeds obtained through selfing of the regenerated plants, allow isolation of tranformed plant lines with a limited number of transgenes in the progeny of the regenerated plants systemically infected with the *Agrobacterium* strains. It is clear that not all progeny seeds necessarily will yield transgenic plants, but methods to detect the presence of transgenes (Southern, PCR, expression of marker genes) are well known in the art.

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It should be appreciated that the above described cloning methods are only applicable to fertile plants. Plants which e.g. cannot undergo gametogenesis will require the use of *in vitro* cloning methods. Several methods are available in the art, including but not limited to protoplasting (e.g. described in Lijsebettens *et al*, 1986, *J. Mol. Biol.* 188, 129-145)

mechanical disruption of the tissue (Meins and Binns, *Proc. Natl. Acad. Sci USA* 74, 2928-2932) or shoot generation from explants such as e.g. leaf discs. It is thought that the inability of the auxotrophic *Agrobacteria* strains present in the plant tissues to grow on the plant media used for *in vitro* culture, allowing the use of *in vitro* cloning systems without having to resort to bacteriocidal or bacteriostatic compounds, constitutes a major advantage of the described transformation processes.

Regenerated plants can be cured of the systemically spread *Agrobacteria*, by culturing the intact regenerated plants in the absence of the required nutrient and in the presence of a bacteriocidal compound, such as an antibiotic. It should be noticed that at this stage no regeneration is any longer required, hence the bacteriocidal compounds can be used despite their potential noxious effects on regeneration. It is also generally thought that *Agrobacterium* does not transmit via seeds. Harvesting progeny seeds thus represents an alternative way to obtain plants cured from *Agrobacteria*.

In accordance with this invention, transformation protocols are provided comprising the further step of stimulating locally the growth of the endemic *Agrobacterium* population, preferably at the site of appropriate target tissues, particularly at the site of inflorescence meristems. To this end, the regenerated plant, systemically infected by the auxotrophic *Agrobacterium* strain, is locally supplied with the required nutrient, particularly with the required methionine, to stimulate locally *Agrobacterium* division and transfer of the DNA of interest to the cells of the target tissue of interest. The mode of application is considered not to be important, and methods for application of chemicals to plants are generally available in the art (e.g. spraying with a solution of the required nutrient in 70 % acetone).

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In a preferred embodiment, the required nutrient is supplied to the cell lineage yielding the gametes and ultimately the seeds, particularly to the inflorescence meristems or immature inflorescences. In another embodiment the required nutrient is supplied to tissue particularly amenable for *in vitro* regeneration.

It is preferred to allow a time period, particularly at least one day, to lapse before proceeding to the cloning step. It is clear that in case inflorescence meristems or immature inflorescences have been supplied with the required nutrient, it is preferred that sufficient time is allowed to lapse to allow harvest of the gametes or the seeds.

It is clear that for the method of the invention, it is not strictly required that the whole plant be infected with the auxotrophic *Agrobacteria*. Plants wherein only parts, such as e.g. leaves, are infected by the auxotrophic *Agrobacteria* can be used as suitable material to isolate the transformed cells for regeneration of the transformed plants according to the method of the invention.

In this regard, it is important to note that a "systemically infected plant" as used herein, is a plant wherein the *Agrobacterium* strain is present in at least some part of the plant, preferably in at least several parts of the plants.

The described *in planta* transformation methods using auxotrophic *Agrobacterium* strains are expected to be suited for transformation of any plant for which regeneration methods from explant tissue are available. The methods of the invention will be especially suited for transformation of plants wherein the transformation and regeneration cannot be achieved in a single step. The *in planta* transformation methods are particularly suited for transformation of sweet pepper (*Capsicum annuum*) cucumber (*Cucumis sativus*), sunflower (*Heliantus annuum*), leek (*Allium*

ampeloprasum), corn (*Zea mays*) , wheat (*Triticum spp*, particularly *T. aestivum* and *T. turgidum*), barley (*Hordeum vulgare*), triticale (*Triticosecale spp.*) oat (*Avena spp*) rye (*Secale cereale*) and rice (*Oriza sativa*).

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It is clear that the auxotrophic *Agrobacterium* strains of the invention can be used in any *Agrobacterium*-mediated plant transformation protocol, thereby obviating the need for control of the proliferation of the *Agrobacterium* with bacteriostatic or bacteriocidal compounds. It is believed that the auxotrophic *Agrobacterium* strains of the invention, preferably methionine requiring *Agrobacterium* strains, particularly LBA4404*met* are especially suited for use in transformation of plant protoplasts, particularly carrot petiole protoplasts, sugarbeet guard cell protoplasts, and corn protoplasts, and for use in transformation of callus tissue, preferably embryogenic callus tissue, particularly embryogenic callus tissue from sugarbeet or cucumber. The auxotrophic *Agrobacterium* strains of the invention are expected to be useful for the transformation all plants, either dicotyledonous or monocotyledonous but are particularly suited for the transformation of sweet pepper (*Capsicum annuum*) cucumber (*Cucumis sativus*), sunflower (*Heliantus annuum*), leek (*Allium ampeloprasum*), sugar beet (*Beta spp*), chicory (*Cichorium spp.*) corn (*Zea mays*) , wheat (*Triticum spp*, particularly *T. aestivum* and *T. turgidum*), barley (*Hordeum vulgare*), triticale (*Triticosecale spp.*) oat (*Avena spp*) rye (*Secale cereale*) and rice (*Oriza sativa*)

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Although not intending to limit the present invention to any one theory or mode of action, it is thought that the reduction in fitness of the auxotrophic bacteria in the used *in vitro* culture media is pivotal for the increased efficiency in transfer by reducing the stress induced by the vigorous proliferation and associated pathogenesis-related processes induced in the plant, yet avoiding the negative effects imposed on the plant cells,

particularly protoplasts, of added bacteriostatic or bacteriocidal compounds.

The following Examples describe the methods of the invention in detail.

5 Unless stated otherwise in the Examples, all recombinant DNA techniques are carried out according to standard protocols as described in Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, NY and in Volumes 1 and 2 of Ausubel *et al.* (1994) *Current Protocols in Molecular Biology*, *Current*
10 *Protocols*, USA. Standard materials and methods for plant molecular work are described in *Plant Molecular Biology Labfax* (1993) by R.D.D. Croy, jointly published by BIOS Scientific Publications Ltd (UK) and Blackwell Scientific Publications, UK.

15 In the examples and in the description of the invention, reference is made to the sequences of the Sequence Listing. The following free text is contained within the Sequence Listing:

<223> Description of Artificial Sequence:T-DNA of pGSV71

20 <223> RB: T-DNA right border

<223> CaMV35S P3 promoter

<223> region encoding phosphinotricin acetyltransferase

<223> 3'nos: 3' untranslated region containing the polyadenylation signal of the nopaline synthase gene of *Agrobacterium* T-DNA

25 <223> LB: T-DNA left border

Strains LBA4404 $_{metHV}$ and ATHV $_{ade,his}$ have been deposited at the Belgian Coordinated Collections of Microorganisms (BCCM), Laboratorium voor Microbiologie - Bacteriënverzameling (LMG), Universiteit Gent, K. L.

Ledeganckstraat 35, B-9000 Gent, Belgium on August 20, 1998, and have been attributed the following deposition numbers:

LBA4404 met HV: LMG P-18486

ATHV ade, his : LMG P-18485

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EXAMPLES

Example 1: Selection of an auxotrophic *Agrobacterium* strain

10 A single colony from a freshly grown *Agrobacterium tumefaciens* strain LBA4404 (on LB medium supplemented with streptomycin 25 μ g/ml) was inoculated in 5 ml of LB broth (supplemented with streptomycin 25 μ g/ml) and grown at 28°C on a rotatory shaker for 48 hrs. 2 ml of this culture was irradiated with X-rays at 600 Krad for 37,5 min or 200 Krad for 12,5 min.

15 To 1 ml of the irradiated culture , 2 ml of minimal M9 medium (60 mM K_2HPO_4 , 33 mM KH_2PO_4 , 0.75 mM $(NH_4)_2SO_4$, 0.17 mM tri-sodium citrate.2 H_2O , 0.02% $MgSO_4$, 0.2% glucose, 0.0005 % thiamine) supplemented with 1 g/l Casamino acids and 25 μ g/ml streptomycin were added , incubated for 48 hrs at 28° C with shaking. These cultures were

20 plated at appropriate dilutions and surviving colonies (about 10^6 CFU/ml) were screened on minimal medium (25 μ g/ml streptomycin) with and without 1g/L Casamino acids. Two colonies were isolated which were unable to grow on unsupplemented minimal medium. These auxotrophic colonies were then used to inoculate a series of 2 ml liquid minimal M9

25 medium cultures supplemented with 0.1 mM of each of the amino acids separately and incubated at 28°C with shaking for about 48 hrs. The first auxotrophic strain grew well on isoleucine and threonine supplemented media and grew slightly on asparagine, phenylalanine or alanine. The second auxotrophic mutant strain grew only on minimal media

30 supplemented with methionine. This latter strain was designated LBA4404 met .

LBA4404*met* was unable to grow on media comprising the direct precursor of methionin, homocystein. It can thus be deduced that the mutation in LBA4404*met* affects homocysteine methyltransferase.

Reversion mutation frequency was estimated by plating dense cultures of LBA4404*met* on minimal medium plates (not supplemented with methionine) and incubating for prolonged time at 28°C. Even after two weeks no colonies were observed on a plate inoculated with 10 µl of undiluted bacterial culture (1.7×10^8 CFU/ml), allowing estimation of the reversion frequency as below 0.5×10^{-7} .

Example 2: Agrobacterium LBA4404*met* mediated transformation efficiencies improved by inclusion of *vir* genes derived from a hypervirulent helper Ti-plasmid.

The nearly isogenic strains LBA4404*met* and LBA4404*met*HV comprising the T-DNA vector pNUN25 were used to evaluate the influence on the transformation efficiency of a hypervirulent helper Ti-plasmid in an auxotrophic *Agrobacterium* strain. Strain LBA4404*met*HV was obtained by introduction of plasmid pTOK47 (Jin *et al.*, *supra*) in LBA4404*met* (by electroporation). pNUN25 is a T-DNA vector, comprising a *nptII* gene under control of a *nos* promoter and operably linked to a *nos* terminator between the T-DNA borders, as well as a bacterially expressed *nptII* gene outside of the T-DNA borders. It was constructed by introducing a DNA fragment comprising the glutamine-synthase coding region from *Nicotiana sylvestris* (having the sequence of the EMBL entry under Accession number X66940 from nucleotide 67 to nucleotide 1363) and to which an *Xba*I site (5' of the start codon) and an *Eco*RV site (3' of the end of the coding region) had been engineered by PCR amplification, into the *Xba*I/ *Eco*ICRI linearized pNUN5 (WO 94/29465).

Nicotiana tabacum leaf discs were incubated for two days in 5 ml MS20 medium [MS salts (Murashige and Skoog, 1968 *Physiol. Plant.* **15**, 473-

497) supplemented with 20g/L sucrose] supplemented with 1 mg/L BAP (benzylaminopurine), and then inoculated with either LBA4404*met*(pNUN25) or LBA4404*met*HV(pNUN25) to a final OD₆₀₀ of 0.05. The co-cultivation was incubated at 27°C for two days under a 16hr/8hr light/dark cycle. Next, the leaf discs were transferred to solidified MS20 medium supplemented with 1 mg/L BAP and 100 mg/L kanamycin. Kanamycin resistant shoots were removed as they emerged and further cultivated on selective medium. About 60 transformed shoots were isolated from about 100 leaf discs when LBA4404*met*(pNUN25) was used and about 223 transformed shoots were isolated from about 100 leaf discs when LBA4404*met*HV(pNUN25) was used.

This allowed to conclude that the *vir* genes of a hypervirulent Ti-plasmid still exerted the same effect in an auxotrophic background as observed in a prototrophic background by Jin *et al.* (*supra*).

Example 3: *In planta* transformation of cucumber (*Cucumis sativa*)

Cucumber seeds of the genotype Gu1665-M were surface sterilized by incubation for 1 min in a 70% ethanol solution, followed by 15 min incubation in a 4% hypochlorite solution. The seeds were rinsed three times in sterile H₂O and grown *in vitro* to plants. 24 internodia were harvested from the axenically grown cells and incubated for 30 min in 10 ml liquid MS20 to which LBA4404*met*HV (pNUN8) bacteria (grown in LB supplemented with 0.1 mM methionine, 100 mg/L kanamycin, 25mg/L streptomycin and 2 mg/L tetracycline) were added to a final OD₆₀₀ of 0.1. pNUN8 is a T-DNA vector similar to pVDH99 (for description see below) wherein *EcoRI/XhoI* DNA fragment comprising the *nptII* chimeric marker gene has been replaced by a DNA fragment comprising the chimeric phosphinotricin acetyltransferase coding region between CaMV35S derived promoter and terminator sequences. The internodia were removed from the bacterial suspension and the excess of bacteria was removed by a

quick blotting on filter paper . Next the inoculated internodia were transferred to MS20 solid medium for regeneration.

5 Healthy looking, regenerated plants were then used to demonstrate the presence of the bacteria in the flowers, as well as the presence of beta-glucuronidase activity in parts of the flowers by histochemical staining.

To demonstrate the presence of the Agrobacteria, 30 flowers from the gnotobiotically grown plants were each incubated in 1 ml of LB. In 28 cases bacterial growth was observed after 2-3 days of incubation.

10 Flowers were also histochemically stained for beta glucuronidase activity according to Jefferson(1987) (*Plant Mol. Biol. Rep.* 5(4): 387-405). In at least six flowers, blue spots indicating GUS activity and thus DNA-transfer in these tissues was observed. Pollen from flowers of these plants are used to pollinate female flowers. Seeds originating from the pollinated
15 female flowers are germinated on phosphinotricin comprising media to obtain transformed plants.

Example 4: *In planta* transformation of tobacco (*Nicotiana tabacum*)

20 Internodia were isolated from axenically grown tobacco plants (SR1) and incubated for 120 min in a suspension (OD₆₀₀ 0.05 /in 30 ml MS20) of LBA4404 Δ metHV (pNUN8) bacteria. Next the internodia were transferred to a solid MS20 medium supplemented with 10 μ M acetosyringone. Young leaves from *in vitro* regenerated plants were subjected to histochemical GUS-staining and blue sectors of different sizes were observed in several
25 leaves. The difference in size of the stained sectors might reflect the entrance the time-point at which the T-DNA entered the precursor cell(s) in the cell lineage. Leafs of the regenerated plants are used as starting material to isolate leaf discs, which are cultivated on PPT-comprising media to obtain transgenic shoots and ultimately transgenic plants (
30 according to the method described by Horsch *et al.*, 1984, *Science* 223:496-498).

Example 5: *In planta* transformation of tomato (*Lycopersium esculentum*)

Internodia were isolated from axenically grown tomato plants (MoneyMaker) and incubated for 15 min in a suspension (OD₆₀₀ 0.05 /in 30 ml MS20) of LBA4404*met*HV (pVDH99) bacteria. pVDH99 is a plasmid comprising a T-DNA with a 35S GUS-intron chimeric gene as described by Van Canneyt *et al.* (1990, *Mol. Gen. Genet.* 220: 245-250) and a chimeric selectable *CaMV35S-nptII* gene. The plasmid further comprises a bacterial kanamycin resistance. Next the internodia were transferred to a solid MS20 medium supplemented with 10 µM acetosyringone. Young leaves from *in vitro* regenerated plants were subjected to histochemical GUS-staining and blue sectors of different sizes were observed in several leaves. The difference in size of the stained sectors might reflect the entrance the time-point at which the T-DNA entered the precursor cell(s) in the cell lineage. Leafs of the regenerated plants are used as starting material to isolate leaf discs, which are cultivated on kanaycin-comprising media to obtain transgenic shoots and ultimately transgenic plants (according to the method described by Mc Cormick *et al.*, 1986 *Plant Cell Reports* 5: 81-84).

Example 6: *In planta* transformation of leek (*Allium ampeloprasum*)

For this purpose, fully mature field grown plants are used and harvested. Plants are left to dry for 3 days in order to reduce infections. Basal plates are collected, cut in four pieces (with complete elimination of the roots) and subsequently sterilized by submersion for 1 minute in 70 % ethanol, followed by 20 minutes treatment with 2% NaOCl (commercial bleach). After this treatment the explants are washed 4 times with sterile water, with 10 min intervals between the washing steps.

These explants are transferred to induction medium [DS macro elements (Dunstan and Short, 1979, *Sci Hort* 112: 37-43), MS microelements (Murashige and Skoog, 1968 *see infra*), FeEDTA, thiamine 10 mg/L,

pyridoxine 1 mg/L, nicotinic acid 1 mg/L, inositol 100 mg/L, sucrose 30g/L, isopentenyl adenine (2-IP) 4 mg/L NAA, NAA 1.25 mg/L, Agar 8 g/L]. After 3 to 4 weeks incubation at 21°C with a light intensity of 2000 to 4000 lux, the first plantlets emerge, which are then used for the inoculation with *Agrobacterium* LBA4404 $\text{met}^{\text{H}}\text{V}$ (pVDH99). To this end, the young plantlets are removed from the initial explants and an incision is made in the basal part of the young plantlets. This wounded basal part is soaked in an *Agrobacterium* suspension with an OD_{600} of about 0.01 for a period of 30 minutes. The excess of *Agrobacterium* suspension is quickly removed by blotting on filter paper, prior to transfer to the induction medium. The inoculated plants develop adventitious plants, some of which are tested by histochemical GUS staining to verify the presence of putative transgenic sectors. Similar adventitious plantlets are transferred to a medium that allows the outgrowth to mature leek plants [similar to the induction medium except that NAA is reduced to 0.1 mg/L and sucrose to 20 g/L. Seeds are harvested from the regenerated mature plants and germinated to obtain transgenic plants. Transgenic plants are recognized by their ability to grow on kanamycin containing media. Transgenic plants are further recognized by PCR detection of the inserted T-DNA sequences.

Example 7: *Agrobacterium* mediated transformation of embryogenic callus from cucumber (*Cucumis sativa*)

Embryogenic callus from cucumber genotype 941687-G was generated as described (Chee, P. (1990) *Hortscience* **25** (7), 792-793). The callus was cut in a petridish into little pieces of less than 1 mm in 5 ml MS30 2/0.5 2-4D/kin [MS salts (Murashige and Skoog, 1968 *Physiol. Plant.* **15**, 473-497) supplemented with 30g/L sucrose, 2 mg/L 2,4-dichlorophenoxy acetic acid (2,4-D) and 0.5 mg/L kinetin]. To this petridish, an *Agrobacterium tumefaciens* LBA4404 $\text{met}^{\text{H}}\text{V}$ (pTOK47) (pVDH99) suspension was added to a final OD_{600} of 0.5. The bacteria had been cultivated for 2 days at 28°C

with shaking, in 10 ml LB supplemented with 0.1 mM methionine, 100 mg/L kanamycin, 25 mg/L streptomycin and 2 mg/L tetracyclin. The bacteria were harvested by centrifugation, washed with the MS30 2/05 2-4D/kin medium and resuspended to the original density.

5 After 4 days of co-cultivation at 27°C in the dark, the calli were washed twice with 25 ml of liquid MS30 2/0.5 medium, plated on solid medium MS30 2/0.5 supplemented with 100 mg/L kanamycin, and further incubated at 27°C in the dark. After two weeks, selected calli were subjected to histochemical GUS-staining as described by Jefferson (1987) (10 *Plant Mol. Biol. Rep.* 5(4): 387-405). Blue sectors could be visualized in at least 5 calli, indicative of cells transformed by the T-DNA of pVDH99. Healthy looking sectors of the calli grown on the selective medium were dissected out and transferred to similar medium without kanamycin, to allow regeneration.

15 **Example 8: Transformation of carrot protoplasts by an auxotrophic *Agrobacterium* strain.**

Carrot protoplasts were isolated from *Daucus carota* var Sytan B142 petioles according to Dirks *et al.*, 1996 (R. Dirks, V. Sidorov, C. Tulmens 20 1996, *Theor. Appl. Genet.* 93: 809-815) resuspended at a density of 8×10^5 protoplasts /ml in CPP-CA (CPP medium according to Dirks *et al.*, 1996 with omission of the casamino acids) supplemented with 0.1 mg/L 2,4-D and 0.2 mg/L zeatin. Four petridishes containing 1.5ml of protoplast suspension were co-cultivated with *Agrobacterium tumefaciens* 25 LBA4404*met* (pNUN20) (added to a final OD₆₀₀ of 0.05) for two days at 27°C. No antibiotics were added to the protoplast culture media. The bacteria were harvested and washed prior to addition to the protoplasts, to remove the methionine.

30 pNUN20 is a T-DNA vector comprising a T-DNA with a chimeric selectable *nptII* gene and a chimeric gene consisting of a CaMV 35S promoter

operably linked to a beta-subunit H⁺ ATPase (Shinozaki, K., Devo, H., Keto, A. and Suginec, M. (1983) *Gene* 24, 147-155 ; EMBL-database accession number K00507) followed by a nopaline synthase gene terminator. The plasmid further comprises a bacterial kanamycin resistance gene outside of the T-DNA borders.

After two days, 1.5 ml of alginate was added to each petridish and the alginate discs containing the protoplasts were floated on 3 ml CPP-CA supplemented with 0.1 mg/L 2,4-D and 0.2 mg/L zeatin, for about 12 days before addition of 100mg/L kanamycin. The discs were further incubated about two weeks. Next, the alginate discs were dissolved in 40 mM sodium citrate, washed, resuspended in 5 ml CPP-CA 30/20 S/N supplemented with 0.1 mg/L NAA (naphtalene acetic acid) and 0.2 mg/L zeatin and plated on solid medium (CPP-CA 30/20 S/N supplemented with 0.1 mg/L 2,4-D and 0.2 mg/L zeatin) containing either 75 or 100 mg/L kanamycin. Calli growing healthy on the selective media were transferred to fresh selective medium as they appeared. About 285 green kanamycin resistant calli were obtained from about 4.8×10^6 protoplasts. These calli were transferred to B5-0.1 medium (B5 medium according to Gamborg, O., Miller, R., Ojima K. (1968) *Experimental Cell Research* 50: 151-158 containing 0.1 mg/l 2,4 D) supplemented with 200 mg/L Claforan and 100 mg/L kanamycin to induce embryogenic calli and B5 medium without growth regulators was used to regenerate plants .

Example 9. Transformation of chicory (*Cichorium sp.*) with an auxotrophic *Agrobacterium* strain.

Seeds of chicory were surface-sterilized (in sodium hypochlorite 2%) rinsed and germinated on MS20 medium and grown at 27 °C for 4 to 8 weeks. Leaf discs of about 4-6 mm diameter were transferred to MSN20 medium [MS salts(Murashige and Skoog, *supra*), vitamins according to Nitsch and Nitsch 1965, *Ann. Phys. Veg* 7, 251-266), glycin 2,0 mg/L,

5 sucrose 20 g/L pH5.8, agar 8g/L) supplemented with 1 mg/L BAP, 0.2 mg/L NAA and 0.01 mM methionine, taking care that the abaxial surface of the leaf discs is in contact with the medium. After 1 day, the leaf discs were submersed for 20 minutes in a bacterial suspension (OD600 between 0.01 - 0.1) of *Agrobacterium tumefaciens* LBA4404 met comprising the T-DNA vector pNUN7. pNUN7 is a plasmid comprising a T-DNA with a CAMV 35S promoter -GUS-intron chimeric gene as described by Van Canneyt *et al.* (*supra*) and a chimeric selectable *nptII* gene. The plasmid further comprises a bacterially expressed kanamycinresistance gene.

10 The leaf discs are further incubated on MSN20 supplemented with 1 mg/L BAP, 0.2 mg/L NAA for two days and then washed in liquid MS20 medium. The washed leaf discs are further incubated on selective medium MNS20 supplemented with 1 mg/L BAP, 0.2 mg/L NAA and 100 mg/L kanamycin (with transfer to fresh selective medium every 14 days). After about 5 weeks the selection medium is changed to MS20 with 0.1 -1 mg/L BAP and 100 mg/L kanamycine.

15 About 20 kanamycin resistant transformed plants were generated from 48 leaf pieces.

20 **Example 10: *Agrobacterium*-mediated transformation of DSM6009 corn protoplasts.**

25 Corn protoplasts of the genotype DSM6009 are prepared according to EP 0 469273 A1, and co-cultivated for 2-3 days with *Agrobacterium tumefaciens* LBA4404 met comprising the helper plasmid pAL4404 and the T-DNA vector pGSV71. pGSV71 is a T-DNA vector derived from pGSC1700 (Cornelissen and Vandewiele, 1989, *Nucl. Acids Res.* 17: 833) differing by the absence of the -lactamase gene and the presence of the T-DNA characterized by the sequence of SEQ ID No. 1. pGV571 30 comprises the selectable chimeric *bar* marker gene, operably linked to a

CaMV35S promoter and the 3' end of the nopaline synthase gene. After 2-3 days, protoplasts are washed with W5 -buffer and cultured further according to EP 0 469273 A1. Selection and regeneration of transformed cells into transformed corn plants are as described in EP 0 469273 A1. 5 Phosphinotricin resistant corn plants are obtained and the presence of the transgene is verified by polymerase chain reaction and southern analysis.

**Example 11: Isolation and evaluation of auxotrophic
Agrobacterium strain ATHVade,his.**

10 An *Agrobacterium* strain auxotrophic for two nutrients was selected by the following protocol, starting from strain ATHV (described by Lazo *et al.*, 1991, *Biotechnology* 9: 963-967 as strain AGL0).

15 Strain ATHV was grown overnight in LB supplemented with 100 µg/ml rifampin (Rif) . One ml of this cell culture was used to inoculate a flask containing 20 ml LB /100 µg Rif , and allowed to grow for 4hrs (with shaking) at 30°C. Ten ml of this culture was illuminated with 254nm UV-light for 2 to 4 hr. 100 µl of the UV-illuminated culture was plated on LB/Rif medium solidified with agar, and incubated in the dark at 30°C for 2 to 3 20 days. The resulting colonies were replica-plated to minimal medium (M9; see Example 1) and LB medium both supplemented with 100 µg/ml rifampin. Colonies unable to grow on the minimal medium were isolated and analyzed for nutrient requirements. One strain, designated ATHVade,his, was identified which required the addition of adenine for 25 growth, and additionally grew much better when histidine was added (see Table 1). This double requirement for exogenously supplied nutrients allows even better growth control of this *Agrobacterium* strain.

Table 1: Growth of *ATHVade,his* in minimal medium with different concentrations of adenine (ade) and histidine (his). The values represented are OD600 measured after two days of growth.

	Ade 0 mM	Ade 0.005 mM	Ade 0.01mM	Ade 0.05 mM	Ade 0.1 mM
His 0 mM	0.019	0.059	0.067	0.111	0.129
His 0.01 mM	0.034	0.07	0.071	0.111	0.141
His 0.05 mM	0.04	0.093	0.112	0.144	0.196
His 0.1 mM	0.035	0.102	0.123	0.163	0.221

5 To analyze whether strain *ATHVade,his* was still capable of T-DNA transfer, a derivative strain containing T-DNA vector pNUN7 (see Example 9) was used to infect leaf discs of *N. tabacum* and *N. sylvestris* in comparison with a wild type ATHV strain containing the same T-DNA vector. In *N. tabacum* about 115 transformed shoots were obtained from about 50 leaf segments after inoculation with ATHV(pNUN7), while about 10 112 transformed shoots were obtained from about 50 leaf segments after inoculation with *ATHVade,his*(pNUN7). In *N. sylvestris* about 17 transformed shoots were obtained from about 50 leaf segments after inoculation with ATHV(pNUN7), while about 40 transformed shoots were 15 obtained from about 50 leaf segments after inoculation with *ATHVade,his*(pNUN7).

Example 12: In planta transformation of *Petunia (Petunia hybrida)*

20

Either internodia or shoot tips (excised below the node bearing the first 3 to 4 mm leaf) were isolated from axenically grown plants and incubated for about 7 to 10 min with *Agrobacterium* LBA4404*met*HV(pNUN7) cell

suspension, blotted dry and cultured on plant culture media, as described in the previous examples. The shoot tips were not immersed but put with the cut end in a well containing the *Agrobacteria*. Internal examination of explants and regenerating plants, at different times, revealed GUS expression associated at least with vascular bundles.

The bacterial presence was estimated in the different parts of the plants after 4 weeks culture *in vitro*. To this end, parts of different leaves were removed, ground in a tube containing 100 μ l LB. After settling, 20 μ l of the supernatants was plated on LB medium containing rifampin. The results are summarized in Table 2.

Table 2. Number of bacteria determined in different infected Petunia plant parts

Tested leaf	# of bacteria in 20 μ l plant extract from nodal cultures (individual exp.)	# of bacteria in 20 μ l plant extract from shoot tip cultures (individual exp.)
Upper tip	204 / 92 / 0	> 200/ >500/0
Upper center	271 / 0 / 0	>500/ 6/ 0
Middle tip	>500/ 5/ 0 / >1000/ >500/ 26/ >1000	200/ 1/ >200/ >1000/ >500/ >100/ 0/ 5/ >1000/ >1000
Middle center	10/ 0/ 50	>200/ 29/ 500
Basal tip	>500/ 449/ 3	> 500/ 19/ >1000
Basal center	>500/ 0/ 0	>2000/ >1000/ 0

All plants tested contained Rif resistant *Agrobacteria*, and about 75% of all the samples tested contained *Agrobacteria*.

Example 13: Comparison of transformation efficiency of *A. tumefaciens* strain LBA4404*met* HV with LBA4404 HV in transformation of *Brassica napus*.

5 The transformation efficiency of *A. tumefaciens* LBA4404*met*HV derivatives (containing T-DNA vectors with a chimeric β -glucuronidase gene) in the transformation of embryo's obtained from microspore cultures of *Brassica napus* "Topas" was compared with the transformation efficiency of LBA4404HV and GV3101 containing similar T-DNA vectors. The
10 sequence of events in the transformation can be summarized as follows:

- a. establishing cell cultures from microspores of *B. napus*
- b. generating somatic embryo's
- c. co-cultivating those embryo's with a *A. tumefaciens* strain
15 comprising the T-DNA vectors
- d. grow the co-cultivated embryo's to the "hypocotyl" stage and inducing secondary embryogenesis.
- e. Submit the secondary embryo's to a selective regime
- f. Score the surviving embryo's for β -glucuronidase expression.

20 When LBA4404*met*HV comprising a T-DNA vector with a chimeric β -glucuronidase gene was used to transform *B. napus* embryo's about 80% of the secondary embryo's obtained had GUS+ sectors, whereas only 5% of the secondary embryo's exhibited such spots when co-cultivated with
25 LBA4404HV comprising a similar T-DNA vector. In a more quantitative assay the transformation efficiency of LBA4404*met*HV; LBA4404HV and GV3101, each comprising a T-DNA vector with a chimeric β -glucuronidase gene was compared. The results, demonstrating a more efficient transformation with LBA4404*met*HV, are summarized in Table 3.

Table 3. Efficiency of transformation, assayed by GUS expression

Host strain	Percentage embryo's with GUS+ sectors	Average number of GUS+ sectors per embryo
LBA4404 <i>met</i> HV	82	>20
LBA4404HV	67	5 -10
GV3101	59	±5

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<p>B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input type="checkbox"/></p>	
<p>Name of depositary institution BCCM - LMG</p>	
<p>Address of depositary institution (including postal code and country) UNIVERSITEIT GENT K.L. Ledeganckstraat 35 B - 9000 GENT, BELGIUM</p>	
<p>Date of deposit August 20, 1998</p>	<p>Accession Number LMG P - 18485</p>
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<p>D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)</p>	
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<p>D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)</p>	
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Claims

- 5 1. A process to produce a transgenic plant comprising a foreign DNA fragment integrated into the genome of at least some of its cells, said process comprising the following steps:
- 10 1) providing a plant which is systemically infected with an auxotrophic *Agrobacterium* strain harbouring a DNA of interest which is operably linked to at least one T-DNA border sequence; and
- 2) generating a transgenic plant from a single cell or a group of cells isolated from said systemically infected plant.
- 15 2. The process according to claim 1, wherein said plant which is systemically infected with said auxotrophic *Agrobacterium* strain is regenerated from explant tissue inoculated with said auxotrophic *Agrobacterium* strain.
- 20 3. The process according to claim 2, wherein said regeneration of said plant which is systemically infected with said auxotrophic *Agrobacterium* strain is performed on a plant regeneration medium, in the absence of a compound with a bacteriocidal or bacteriostatic effect on said auxotrophic *Agrobacterium* strain.
- 25 4. The process according to any one of the claims 1 to 3, wherein said transgenic plant is generated by in vitro regeneration of a protoplast isolated from said systemically infected plant.
- 30 5. The process according to any one of the claims 1 to 3, wherein said transgenic plant is generated by in vitro regeneration of an explant tissue isolated from said systemically infected plant.

6. The process according to any one of the claims 1 to 3, wherein said transgenic plant is generated by in vitro regeneration of a microspore of said systemically infected plant.
- 5 7. The process according to any one of the claims 1 to 3, wherein said transgenic plant is generated by germination of transformed progeny seed of said systemically infected plant.
- 10 8. The process according to any one of claims 1 to 7, which further comprises the step of applying the required nutrient to at least part of the systemically infected plant prior to the step of generating a transformed plant.
- 15 9. The process according to claim 8, wherein said required nutrient is applied to the inflorescence meristem or immature inflorescence of said systemically infected plant.
- 20 10. The process according to any one of claims 1 to 9, wherein said *Agrobacterium* is auxotrophic for cysteine or methionine or for adenine and histidine.
- 25 11. The process according to any one of claims 1 to 9, wherein said *Agrobacterium* is LBA4404*met* or ATHV*ade, his*.
12. Bacterial strain LBA4404*met*.
13. Bacterial strain ATHV*ade, his*.

14. The use of an *Agrobacterium* strain auxotrophic for methionine or cysteine or for adenine and histidine in *Agrobacterium*-mediated transformation of plants.
- 5 15. The use of *Agrobacterium* strain auxotrophic for methionine or cysteine or for adenine and histidine in *Agrobacterium* mediated transformation of callus from sugarbeet .
- 10 16. The use of *Agrobacterium* strain auxotrophic for methionine or cysteine or for adenine and histidine in *Agrobacterium* mediated transformation of corn protoplasts.
- 15 17. The use according to any one of claims 13 to 15, wherein said auxotrophic *Agrobacterium* is selected from the group of LBA4404*met*, ATHV*ade, his* and derivatives thereof.

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 98/05372

A. CLASSIFICATION OF SUBJECT MATTER IPC 6 C12N15/82 C12N1/20 A01H5/00				
According to International Patent Classification (IPC) or to both national classification and IPC				
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC 6 C12N A01H C12R				
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched				
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)				
C. DOCUMENTS CONSIDERED TO BE RELEVANT				
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.		
A	SCHMIDT, R.M., ET AL. : "growth requirements and infectivity of auxotrophic adenine-dependent mutants of Agrobacterium tumefaciens". PHYTOPATHOLOGY, vol. 59, no. 10, 1969, pages 1451-1454, XP002087322 see the whole document	1-17		
A	WO 95 34667 A (DANISCO A/S) 21 December 1995 see page 4	1-17		
A	WO 91 13159 A (BIOSEM) 5 September 1991 see the whole document	1-17		
--- -/-- ---				
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C.				
<input checked="" type="checkbox"/> Patent family members are listed in annex.				
* Special categories of cited documents :				
<table style="width: 100%; border: none;"> <tr> <td style="width: 50%; border: none; vertical-align: top;"> "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed </td> <td style="width: 50%; border: none; vertical-align: top;"> "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "&" document member of the same patent family </td> </tr> </table>			"A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "&" document member of the same patent family
"A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "&" document member of the same patent family			
Date of the actual completion of the international search <p style="text-align: center;">9 December 1998</p>		Date of mailing of the international search report <p style="text-align: center;">28/12/1998</p>		
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016		Authorized officer <p style="text-align: center;">Holtorf, S</p>		

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 98/05372

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DATABASE WPI Section Ch, Derwent Publications Ltd., London, GB; Class B05, AN 72-22820T XP002055372 & JP 47 010999 B (KYOWA HAKKO KOGYO CO LTD) see abstract -----	1-17

INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No

PCT/EP 98/05372

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9534667 A	21-12-1995	AU 690999 B	07-05-1998
		AU 2669495 A	05-01-1996
		BR 9507970 A	12-08-1997
		CA 2192552 A	21-12-1995
		EP 0766743 A	09-04-1997
		JP 10501139 T	03-02-1998
		NZ 287848 A	26-06-1998
		PL 317578 A	14-04-1997
WO 9113159 A	05-09-1991	FR 2658987 A	06-09-1991
		FR 2658988 A	06-09-1991
		AT 129745 T	15-11-1995
		DE 69114275 D	07-12-1995
		DE 69114275 T	13-06-1996
		DK 517833 T	04-12-1995
		EP 0517833 A	16-12-1992
		ES 2079647 T	16-01-1996